Pharmaceutical product for the treatment of viral
infections, in particular of the human immunodeficiency
virus (HIV)

        The present invention relates to a pharmaceutical
product for the treatment of viral infections, in
particular of the human immunodeficiency virus (HIV).
        Viral infections, in particular infections by the
5 human immunodeficiency virus (HIV) are the serious threat
to public health. World-wide much effort goes into the
development of a pharmaceutical product to combat this
usually incurable disease.
        It is known that iron is involved in the
10 replication of human immunodeficiency virus. In the first
place, the HIV-1 long terminal repeat (LTR) portion
contains nuclear factor (NF)-kB response elements that
regulate proviral transcription. NF-kB activation may be
influenced with iron via the production of reactive oxygen
15 species.
        A second route by which iron chelation can
influence HIV replication is by inhibition of the DNA
synthesis by inactivation of iron-dependent ribonucleotide
reductase.
20   Another strategy which can be employed in
targeting iron chelators against HIV-1 is a direct viral
DNA/RNA attack.
        It is the object of the present invention to
provide a pharmaceutical product for the efficient
25 treatment of viral infection, in particular of the human
immunodeficiency virus, to which end the pharmaceutical
product is characterized in that the pharmaceutical
product comprises a compound that as active component
contains an iron chelator.
30   Suitable iron chelators according to the
invention may be selected from the group of hydroxamates
(such as deferoxamine), the family of the

hydroxypyridinons (such as deferiprone), and the nucleic acid-binding chemotherapeutical products (such as bleomycin).

5   Very surprisingly it has been shown that in vitro particularly favourable results were obtained when in addition to the component containing as the active compound an iron chelator, a preparation containing another virus-inhibiting compound was used; which was due to a synergistic effect. A protease inhibitor, in

10 particular ritonavir, proved to be especially suitable as the antiviral agent.

    Another antiviral agent producing a favourable synergistic effect with iron chelators is reverse transcriptase inhibitor, in particular dideoxyinosine.

15   In this connection applicant has examined the effect of deferoxamine (DF), deferiprone (L1) and bleomycin (BLM) on HIV-1 replication in primary blood cells. For this purpose human peripheral blood lymphocytes (PBL) and monocyte-derived macrophages (MDM), the main

20 target cells for HIV, were used. It was shown, that the considerable production of HIV-1 replication by DF was accompanied by a proliferation inhibition. The same was observed with L1. Surprisingly, BLM inhibited the viral replication in PBL and MDM without affecting lymphocyte

25 proliferation. The use of iron chelators in combination with established antiviral agents for antiviral combination therapy was examined. When DF was used in combination with the nucleoside analog dideoxyinosine (ddI), a surprising synergistic inhibition of p24 in HIV-

30 infected PBL seemed to occur.

    <u>Materials and methods used in the study are the</u> <u>following</u>.

    Cell isolation.

35   Peripheral Blood Mononuclear Cell (PBMC) fractions were isolated from heparinized blood from HIV-1-, HIV-2- and hepatitis B-seronegative donors (Bloodbank, Utrecht, The Netherlands) by Ficoll-Isopaque

gradient separation. Cells were washed twice and were then either subjected to counter-current centrifugal elutriation, or monocytes in the PBMC fraction were allowed to adhere to fibronectin-coated flasks before the

5 PBL fraction was harvested. Monocytes were of >95% purity by criteria of cell morphology on May-Grünwald-Giemsa-stained cytosmears and by non-specific esterase staining using (α-naphthylacetate (Sigma Chemical Co., St. Louis, MO) and PBL >85% purity by May-Grünwald-Giemsa-staining.

10 Viability of both cell types was >95% at the point of experiment initiation as determined by trypan-blue exclusion. Isolated monocytes were cultured in suspension at a concentration of 2×10^6 cells/ml in teflon flasks minimizing adherence (Nalgene, Rochester, NY) and allowed

15 to differentiate for 7 days. MDM medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated human AB serum, negative for anti-HIV antibodies (Bloodbank Utrecht, NL), 10 μg/ml gentamycin (Life Technologies Ltd, Paisley, Scotland), 5 μg/ml

20 ciprofloxacin (Beyer, Leverkusen, Germany), 0.6 mg/ml L-glutamine (Sigma) and 3.4 g/L bicarbonate (Merck, Darmstadt, Germany). Isolated PBL was stimulated to proliferate for 3 days with 4 μg/ml phytohemagglutinin (PHA; Sigma) in RPMI-1640 (Life Technologies Ltd.) medium

25 supplemented with 10% heat inactivated fetal calf serum (Life Technologies Ltd.) and 10 μg/ml gentamycin. After PHA stimulation the PBL were cultured in medium containing 10 U/ml human recombinant IL-2 (Boehringer, Mannheim, Germany). All incubations were carried out in flat-

30 bottomed 96-well plates at 37 °C, 5% CO_2 and 95% air.

HIV infection and p24 measurements.

Cells were infected for 2 hours with HIV-1$_{Ba-L}$. Macrophages were infected with a multiplicity of infection

35 (MOI) of 0.005 and lymphocytes with an MOI of 0.001. The cells were then washed twice to remove excess virus and subsequently incubated with either DF (Novartis Pharma, Arnhem, The Netherlands), Ll (from Duchefa Farma B.V.,

Haarlem, The Netherlands) or BLM (H. Lundbeck A/S,
Copenhagen, Denmark) for a period of 5 days. Virus in
culture supernatant was inactivated in a final
concentration of 0.05% empigen (Calbiochem-Novabiochem

5   Co., La Jolla, CA). The presence of HIV-1 in the
inactivated supernatant was monitored by checking for the
p24-core antigen by enzyme-linked immunosorbent assay
(ELISA).

10   *Lymphocyte proliferation measurements.*
           Since iron chelators may influence cellular
proliferation the effect of the three iron chelators on
PBL proliferation was also determined. This was performed
by monitoring overnight ^{3}H-thymidine (^{3}H-TdR) incorporation

15   into DNA. 25 μl ^{3}H-TdR (0.5 μCi, Amersham International
plc, UK) was added in the presence of DF, L1, BLM or
DF/ddI combination, in a total volume of 200 μl cell
suspension after 4 days incubation with the different
compound concentrations. Cell incubations were in 96-well

20   plastic plates (Costrar, Cambridge, MA, US). After
overnight incubation with ^{3}H-TdR, the cells were harvested
onto a filter using an automatic cell microharvester
(Titertek® cell harvester 530). The filter was
subsequently dried and immersed in a scintillation

25   cocktail (Wallac UK). Radioactivity was counted in a Mark
II liquid Scintillation system (Model x, Tricton
analytics, Inc.).

           Cytotoxicity.

30   Possible cytotoxicity of DF, L1 or BLM was
monitored by the MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide] (Sigma) assay.

           Studies of DF-ddI combination.

35   Three separate experiments were performed
substantially as described in the section *HIV infection
and p24 measurements*. Multiply-diluted fixed-ratio
combinations of the drugs, or single drugs were added to

each incubation point. The IC_{50} (inhibitory concentration)
of each drug was calculated using the computer software
program CalcuSyn. The combined-drug effect was evaluated
by the median-effect principle and the isobologram method.
5   This method involves the plotting of the dose-effect curve
for each drug alone and in combination. The slope of the
median-effect plot and the x intercept of the plot were
then used for the computerized calculation of a
combination index (CI). The CI values were based on the
10 classic isobologram equation and CI values of <1, 1 and >1
indicate synergism (greater than the expected additive
effect), additive effects and antagonism (less than the
expected additive effect). Fraction affected (f_a) was
calculated for every drug combination and for each drug
15 alone after p24 measurements in infected PBL incubated for
5 days with the examined compounds (see *HIV infection and
p24 measurements*).

Statistical analysis.
20   Repeated measures ANOVA and student Newman-Keuls
test were used to analyze the data. *P* values below 0.05
were considered significant.

Results
25   *Effect of DF on HIV-1 replication, cellular
proliferation and cytotoxicity in PBL.*
DF was shown to reduce p24 levels in culture
supernatants. At a concentration of 3 μM DF a 40%
reduction was observed in viral replication in PBL (19 vs
30 11 ng p24/ml, n=3, p<0.05). A compound concentration of 30
μM reduced p24 levels by >90% (figure IA) from 19 to 1.9
ng p24/ml (n=3). To rule out the possibility that these
inhibitory effects were due to cytotoxicity, cell
viability was monitored. A DF concentration of 100 μM was
35 found to be cytotoxic (figure IB). In addition, due to the
anti-proliferative properties of DF, cellular
proliferation was monitored. The observed reduction in p24
was mirrored by a decrease in proliferation (figure IC).

Thus, non-cytotoxic DF concentrations were capable of reducing HIV replication and this reduction was accompanied by cellular proliferation inhbition.

5   *Effect of L1 on HIV-1 replication, cellular proliferation and cytoloxicity in PBL.*
       HIV-1 replication in lymphocytes was reduced to minimal levels by 100 μM L1 (figure 2A). This concentration was not cytotoxic in PBL (figure 2B). There
10   seemed to be a threshold point in L1 concentration where the inhibition slope became very steep and this phenomenon was also mirrored by a decrease in proliferation (figure 2C). Thus, the HIV-inhibition observed with L1 could also be attributed to cellular proliferation inhbition. At
15   lower compound concentrations a subtle increase in p24 antigen production was observed (figure 2A).

        Effect of BLM on HIV-1 replication, cellular proliferation and cytotoxicity in PBL.
20   BLM dose-dependently decreased HIV-1 replication in lymphocytes (figure 3A). This compound was not cytotoxic in PBL (figure 3B). At concentrations in the range of 3-300 ng BLM/ml a 20-50% reduction was noted, reflecting a decrease from 19 to 15 (20%) or 9.5 (50%) ng
25   p24/ml (n=3) (figure 3A). Cellular proliferation remained intact (figure 3C). Thus, HIV-1 inhibition by BLM, in contrast to DF and L1, seems to proceed via a pathway other than cellular proliferation inhibition.

30   *Effect of DF, L1, and BLM on HIV-1 replication and cytotoxicity in MDM.*
        In MDM, DF concentrations around 100 μM reduced viral replication by up to 50% (1.2 vs 0.6 ng p24/ml) in a dose-dependent manner (figure 4AI). However this p24
35   decrease was associated with the onset of cytotoxicity (figure 4AII). L1 in the concentrations studied, strikingly up-regulated p24 antigen production by up to

two fold at 30 μM (figure 4BI). These concentrations were not cytotoxic (figure 4BII).

In MDM a dose-dependent decrease in p24 levels was also observed with a 50% reduction at 300 ng BLM/ml
5 from 1.2 to 0.6 ng p24/ml (figure 4CI). BLM concentrations, similarly to PBL, were found not to affect cell viability in MDM (figure 4CII).

Combined antiviral effects of DF-ddI.

10   Moderate to strong synergism was detected when DF was used in combination with ddI. Figure 5 shows a representative graph of the combination index (CI) with respect to fraction affected (f_a) for the inhibition of HIV by a mixture of DF and ddI (molar ratio 4:1). IC_{50} of DF
15 was 8.5 μM and that of ddI 3.7 μM. Figure 5 clearly shows that there is a marked synergism at all f_a values obtained with the different combinations, with the synergism getting stronger as f_a increases. At around the IC_{50} concentrations of both DF and ddI (combination points on
20 figure 5: 10 μM DF: 2.5 μM ddI), the highest f_a value was obtained and the combination was strongly synergistic. Cellular proliferation was also monitored with these combinations and with each drug alone. It was found that ddI in combination with DF did not have an additional
25 inhibitory effect on cellular proliferation when compared to DF alone (results not shown).

The invention is explained in more detail with reference to the Figures 1-6.

Fig. 1. Effect of DF on HIV-I replication (A),
30 cytotoxicity (B) and cellular proliferation (C) in PBL after 5 days incubation with DF.

For the HIV-1 replication measurements cells were infected for 2 hours with HIV-1$_{Ba-L}$ at an MOI of 0.001; the cells were subsequently washed 2 times and incubated with
35 different DF concentrations. After 5 days, supenatant was harvested for p24 ELISA. Virus replication was expressed as % p24 reduction after DF incubations, compared to p24 measured from control HIV-infected cells incubated in the

absence of DF (set at 100% p24). Cytotoxicity was measured
by the MTT test and is represented as % viable cells after
5 days DF incubations, compared to viable cells from
control HIV-infected cells incubated in the absence of DF

5   (set at 100% viable cells). PBL proliferation was measured
by overnight ³H-thymidine incorporation after DF was
incubated for 4 days with cells; it is represented as
counts per minute (cpm) after DF incubations, compared to
cpm from control cells, incubated in the absence of DF.

10   Results are the average of 3 experiments in duplicate
(statistical analysis using repeated ANOVA and student
Newman-Keuls test, *p<0.05). 100% p24 production
represents 19 ng p24/ml

Fig. 2. Effect of L1 on HIV-1 replication (A),

15   cytotoxicity (B) and cellular proliferation (C) in PBL
after 5 days incubation with L1.

For the HIV-I replication measurements cells were
infected for 2 hours with HIV-1$_{Ba-L}$ at an MOI of 0.001; the
cells were subsequently washed 2 times and incubated with

20   different L1 concentrations. After 5 days, supernatant was
harvested for p24 ELISA. Virus replication was expressed
as % p24 reduction after L1 incubation, compared to p24
measured from control HIV-infected cells incubated in the
absence of L1 (set at 100% p24). Cytotoxicity was measured

25   by the MTT test and is represented as % viable cells after
5 days L1 incubations, compared to viable cells from
control HIV-infected cells incubated in the absence of L1
(set at 100% viable cells). PBL proliferation was measured
by overnight ³H-thymidine incorporation after L1 was

30   incubated for 4 days with cells; it is represented as
counts per minute (cpm) after L1 incubations, compared to
cpm from control cells, incubated in the absence of L1.
Results are the average of 3 experiments in duplicate
(statistical analysis using repeated ANOVA and student

35   Newman-Keuls test, *p<0.05). 100% p24 production
represents 19 ng p24/ml.

Fig. 3. Effect of BLM on HIV-1 replication (A), cytotoxicity (B) and cellular proliferation (C) in PBL after 5 days incubation with BLM.

For the HIV-1 replication measurements cells were
5   infected for 2 hours with HIV-1$_{BA-L}$ at an MOI of 0.001; the cells were subsequently washed 2 times and incubated with different BLM concentrations. After 5 days, supernatant was harvested for p24 ELISA. Virus replication was expressed as % p24 reduction after BLM incubations,
10   compared to p24 measured from control HIV-infected cells incubated in the absence of BLM (set at 100% p24). Cytotoxicity was measured by the MTT test and is represented as % viable cells after 5 days BLM incubations, compared to viable cells from control HIV-
15   infected cells incubated in the absence of BLM (set at 100% viable cells). PBL proliferation was measured by overnight ^{3}H-thymidine incorporation after BLM was incubated for 4 days with cells; it is represented as counts per minute (cpm) after BLM incubations, compared to
20   cpm from control cells, incubated in the absence of BLM. Results are the average of 3 experiments in duplicate (statistical analysis using repeated ANOVA and student Newman-Keuls test, *p<0.05). 100% p24 production represents 19 ng p24/ml
25   Fig. 4. Effect of DF (AI-II), L1 (BI-II) and BLM (CI-II) on HIV-1 replication (A-CI) and cytotoxicity (A-CII) in MDM after 5 days incubation.

For the HIV-1 replication measurements cells were infected for 2 hours with HIV-1$_{BA-L}$ at an MOI of 0.001; the
30   cells were subsequently washed 2 times and incubated with different compound concentrations. After 5 days supernatant was harvested for p24 ELISA; Virus replication was expressed as % p24 reduction after compound incubations, compared to p24 measured from control HIV-
35   infected cells incubated in the absence of compounds (set at 100% p24). Cytotoxicity was measured by the MTT test and is represented as % viable cells after 5 days compound incubations, compared to viable cells from control HIV-

infected cells incubated in the absence of compounds (set at 100% viable cells). Results are the average of 3 experiments in duplicate (statistical analysis using repeated ANOVA and student Newman-Keuls test, *p<0.05).

5   100% p24 production represents 1.2 ng p24/ml.

Fig. 5. Graphical presentation of the combination index (CI) with respect to fraction affected (fa) for the p24-antigen inhibition by a mixture of DF and ddI (molar ratio 4:1). Data points on the graph are indicated as conc

10 DF and ddI, both in μM. CT < 1, = 1 and > 1 represent synergistic, additive and antagonistic effects, respectively. The subdivision within the synergistic area (CI >0.7, 0.3-0.7 and <0.3 implying moderate synergism, synergism, and strong synergism respectively) is according

15 to Chou and Talalay. The parameters are obtained using the median effect equation of Chou and Talalay and the CalcuSync computer software programme as described in materials and methods. The IC_{50} within this experiment of DF was 8.5 μM and that of ddI was 3.7 μM. This graph is

20 representative of 3 independent experiments.

Fig. 6. Graphical presentation of the combination index (CI) with respect to fraction affected (fa) for the p24-antigen inhibition by a mixture of bleomycin (BLM) and ritonavir (ratio 30:1). Peripheral blood lymphocytes (PBL)

25 were infected for 2 hours with HIV-1$_{BA-L}$ at an MOI of 0.001. The cells were subsequently washed 2 times to remove an excess in virus and subsequently incubated for 5 days with only BLM, only ritonavir or various combinations of the two substances. Virus in the supernatant of the cell

30 cultures was inactivated in a final concentration of 0.05% empigen and the presence of HIV-1 in the inactivated supernatant was measured by determining the p24 antigen with enzyme-linked immunosorbent assay (ELISA). CI<1, = 1 and >1 indicate synergistic, additive and antagonistic

35 effects, respectively, according to Chou and Talalay. The parameters are obtained using the median effect equation of Chou and Talalay, and the CalcuSync computer software programme. In this experiment the IC_{50} of BLM was 1.9 μg/ml

and of ritonavir 0.04 μM. This graph is representative of two independent experiments.